Construction of nontoxigenic mutants of nonproteolytic *Clostridium botulinum* NCTC 11219 by insertional mutagenesis and gene replacement

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ABSTRACT

Group II nonproteolytic *Clostridium botulinum* (gIICb) are an important concern for the safety of minimally processed ready-to-eat foods, because they can grow and produce botulinum neurotoxin during refrigerated storage. The principles of control of gIICb by conventional food processing and preservation methods have been well investigated and translated in guidelines for the food industry but in contrast, the effectiveness of emerging processing and preservation techniques has been poorly documented. The reason is that experimental studies with *C. botulinum* are cumbersome because of biosafety and biosecurity concerns. In the present work, we report the construction of two nontoxigenic derivatives of the type E gIICb strain NCTC 11219. In the first strain, the botulinum toxin gene (*bont/E*) was insertionally inactivated with a retargeted intron using the ClosTron system. In the second strain, *bont/E* was exchanged for an erythromycin resistance gene using a new gene replacement strategy that makes use of *pyrE* as a bidirectional selection marker. Growth under optimal and stressed conditions, sporulation efficiency and spore heat resistance of the mutants were unaltered except for a small difference in spore heat resistance at 70 °C and in growth at 2.3% NaCl. The mutants described in this work provide a safe alternative for basic research as well as for food challenge and process validation studies with gIICb. In addition, this work expands the clostridial genetic toolbox with a new gene replacement method that can be applied to replace any gene in gIICb and other clostridia.

IMPORTANCE

The nontoxigenic mutants described in this work provide a safe alternative for basic research as well as for food challenge and process validation studies with psychrotrophic *Clostridium*

35 *botulinum*. In addition, this work expands the clostridial genetic toolbox with a new gene
36 replacement method that can be applied to replace any gene in clostridia.

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INTRODUCTION

Botulism is a rare but severe paralytic illness in humans and animals, caused by the botulinum neurotoxin (BoNT) produced by *Clostridium botulinum*. Botulinum toxins are 150 kDa proteins with zinc endopeptidase activity, consisting of two subunits, a 100 kDa heavy chain and a 50 kDa light chain. The heavy chain is responsible for binding and translocation of the light chain into the cytosol of neuronal cells, whereas the light chain cleaves SNARE proteins that are involved in docking of acetylcholine-containing vesicles and fusion to the presynaptic membrane. When the SNARE proteins are cleaved, neurotransmitter release is inhibited, leading to paralysis of the corresponding muscle (1, 2).

C. botulinum is a strictly anaerobic bacterium that thrives in decaying organic matter in soils and sediments of ponds, lakes and oceans. It also forms dormant endospores that are highly resilient to hostile conditions and therefore can be found widespread in the environment. These spores may contaminate foods via the raw materials or other sources, possibly leading to foodborne botulism when they are not eliminated by processing and when their outgrowth is not controlled (3, 4, 5, 6, 7). *C. botulinum* is divided into four distinct groups (I to IV) based on phylogenetic and physiological characteristics, of which only group I and II are typically associated with human botulism. Group I *C. botulinum* consists of proteolytic mesophilic strains producing toxin types A, B and/or F. These strains form heat resistant spores and are the main target for the so-called "botulinum cook", the process used in canning of low acid foods (121 °C/3 min or equivalent). Group II *C. botulinum* (gIICb) comprises nonproteolytic strains producing toxin types B, E or F, which are saccharolytic, psychrotrophic (with minimum growth temperatures of 3 °C) and whose spores are less heat resistant than those of group I (8). In the food industry, gIICb is a major concern for the safety of minimally processed chilled ready-to-eat foods with an

61 extended shelf life (REPFEDs), because spores surviving the mild processing treatments may
62 subsequently germinate, grow out and produce toxin during refrigerated storage (6, 7, 9, 10, 11).
63 The combination of the heat resistance of its spores and the ability to grow under refrigeration
64 conditions makes this pathogen to be the main target pathogen that must be controlled in these
65 foods. Guidelines for control of this hazard in the food industry have been developed more than
66 two decades ago, requiring either a 6-decimal (6-D) reduction of the spores by heat treatment (90
67 °C/10 min or equivalent), or control of outgrowth by limitation of the refrigerated storage time to
68 < 10 days, inclusion of ≥ 3.5% NaCl, ≥ 100 ppm nitrite, acidifying to pH ≤ 5.0 or an appropriate
69 combination of these or additional hurdles (11, 12). However, the increasing consumer's demand
70 for fresh-tasting healthy ready-to-eat foods that have been minimally processed, contain less salt
71 and no artificial preservatives, yet have a long shelf-life, represent a challenge for the food
72 industry in view of these botulinum safety guidelines (10, 11, 12). Furthermore, novel food
73 processing and preservation technologies (e.g. high pressure or pulsed electric field treatment,
74 natural preservatives) have found their way to commercial food production, but data regarding
75 their efficiency to control gIICb is scarce or lacking (13, 14, 15, 16, 17). This is in sharp contrast
76 to the attention given in this context to other pathogens like *Listeria monocytogenes, Salmonella,*
77 enterohemorrhagic *Escherichia coli.* The main reason for this paucity of data is that *C. botulinum*
78 studies are subject to important biosafety and bioterrorism restrictions, and because their culture
79 requires strict anoxic conditions. One possible approach to circumvent these difficulties is the use
80 of nonpathogenic surrogate organisms. For example, *C. sporogenes* has been widely used as a
81 surrogate for proteolytic *C. botulinum* in the studies on thermal processing of low-acid shelf-
82 stable foods (18). However, a suitable validated surrogate for gIICb is lacking up to date.
83 Recently, Parker *et al.* (19) studied previously isolated natural nontoxigenic *Clostridium* spp. that

could possibly be used as surrogates for gIICb. Two out of the three strains analysed showed equal or faster growth than toxigenic gIICb under most (but not all) stress conditions (low temperature, reduced pH and a_w), making them potentially useful for challenge studies with refrigerated foods, at least under some conditions. However, the spores of the three strains had lower heat resistance than some gIICb strains, and thus could not be used for validation of heating processes. Furthermore, it could not be excluded that the strains still carried *bont* or other toxin genes, and the (phylo)genetic relatedness of the strains to gIICb was not documented. This makes the behaviour of the strains under other than the tested environmental conditions unpredictable (e.g. sensitivity to nitrite and other preservatives, sensitivity to organic acids…).

As an alternative approach to develop safe strains for challenge studies and process validation, we attempted in the present work to construct nontoxic derivatives from a toxic gIICb strain by making a targeted knockout of the *bont* gene. The resulting derivative strains are then expected to differ from their parent only in toxin production, and thus to be a highly reliable alternative for challenge studies. Although the inactivation of specific genes in clostridial species has proven to be a rather difficult, slow and inefficient task for a long time, the genetic toolbox for knockout mutagenesis has been expanding the last few years. The ClosTron system, which makes use of a mobile intron that can be retargeted to a sequence of interest, has proven to be particularly efficient for insertional mutagenesis in a range of clostridial species (20), but only a few studies have used ClosTron mutagenesis in gIICb thus far (21, 22). Interestingly ClosTron has been used to knockout the *bont* gene both in gICb and gIICb (21, 23), but the properties of the gIICb knockout strain in view of its possible usefulness for challenge or process validation studies in foods were not further investigated. Importantly, a disadvantage of ClosTron mutagenesis is that reversion to the toxigenic state by excision of the intron from the *bont* gene cannot be excluded.

For this reason, we additionally developed a method for *bont* deletion that makes use of *pyrE*, a gene encoding the enzyme orotate phosphoribosyltransferase which is required for *de novo* uracil biosynthesis. This gene has been used before in various clostridia (*C. sporogenes*, *C. difficile*, *C. acetobutylicum*) as a positive/negative selection marker. PyrE is essential for growth in the absence of uracil, while cells lacking PyrE become resistant to 5-fluoroorotic acid (5-FOA), a substrate analogue in *de novo* uracil biosynthesis that is converted to a toxic product. Therefore, cells encoding *pyrE* can be selected on uracil-free medium or counterselected in the presence of 5-FOA (24, 25, 26). We used this bidirectional selection to delete *bont/E* and replace it by an erythromycin resistance cassette. This is the first report of a targeted gene deletion in gIICb. In addition, our gene replacement strategy is novel in that it allows a single-step selection of gene replacement by double homologous recombination and loss of the plasmid on which the donor DNA is supplied.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Cultures of *C. botulinum* type E NCTC 11219 (obtained from National Collection of Type Cultures, Public Health England) were routinely grown at 30 °C in trypticase peptone glucose yeast extract broth (TPGY; 50 g/l trypticase (BD), 5 g/l bacteriological peptone (Oxoid), 20 g/l yeast extract (Oxoid), 4 g/l glucose, 1 g/l sodium thioglycollate) and plated on reinforced clostridial medium (RCM (VWR), 37 g/l RCM + 15 g/l agar), TPGY agar (TPGY broth + 15 g/l agar) or tryptone yeast extract thioglycollate agar (TYG; 30 g/l tryptone (Lab M), 20 g/l yeast extract, 1 g/l sodium thioglycollate, 15 g/l agar). Trypticase glucose yeast broth (Bio-Rad) supplemented with 0.1% trypsin (Life Technologies) was used to grow cultures for the mouse

bioassays. Uracil-deficient medium was accomplished by replacing yeast extract in TPGY agar with 20 g/l Acid Hydrolysed Casein (Lab M) (27). Clostridial vegetative cultures were manipulated and incubated in a Whitley DG250 anaerobic workstation (initial gas mixture comprised of 80% N_2, 10% CO_2, and 10% H_2) using overnight pre-reduced media. *E. coli* strains were grown in lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) or on LB agar (LB + 15 g/l agar) at 37 °C. *E. coli* DH5α was used for cloning and maintenance of plasmids, while *E. coli* CA434 (HB101 containing plasmid R702, (28)) was used as conjugation donor. Media were supplemented with the following antibiotics (Applichem): thiamphenicol (Tm, 15 μg/ml in agar, 7.5 μg/ml in broth) and erythromycin (Em, 2.5 μg/ml) for *C. botulinum*, and cycloserine (Cy, 250 μg/ml) and chloramphenicol (Cm, 25 μg/ml in agar, 12.5 μg/ml in broth) for *E. coli*. 5-Fluoroorotic acid (5-FOA, 500 μg/ml (Manchester Organics)) was used for the screening of uracil auxotrophs.

Sporulation and purification of spore crops

Spore crops were prepared using a two-phase sporulation medium as described before, with minor adjustments (29). First, a single colony was inoculated in 1 mL TPGY broth at 30 °C. After 24h of growth, this culture was added to a two-phase medium consisting of 4 mL distilled deoxygenated water over solid sporulation medium (3 g cooked meat medium (Oxoid), 0.03 g glucose, 0.45 g agar in 30 mL water). Spores were harvested from the liquid phase after 6 days incubation at 30 °C by centrifugation (3400 × *g*, 4 °C, 15 min). The resulting pellet was washed four times with 0.85% sterile saline by centrifugation, concentrated fivefold and stored in saline at 1-4 °C outside the anaerobic workstation.

151 **Construction of plasmids**

152 The ClosTron technology was used for the generation of an insertion mutant. This system is
153 based on retargeting a bacterial group II intron to insert in a gene of interest (20, 30). Possible
154 target sites in the *bont/E* gene of *C. botulinum* NCTC 11219 were identified using an intron
155 design tool on the ClosTron website (www.clostron.com), and one site was chosen based on a
156 high score according to the algorithm as well as proximity to the N-terminus of the toxin. The
157 plasmid pMTL007C-E2:Cbo:*bontE*-211a, containing the intron flanked by the specific targeting
158 sequences (GGTCATAATAACTACTATCTCCATTTTTTA<intron>CTAATTATTTACAAA)
159 was obtained from DNA 2.0 Inc. (Menlo Park, California, USA).

160 The second knockout strategy consisted of an allelic exchange of *bont/E* with *ermB*, an Em
161 resistance gene. A novel three-step strategy was designed, with each step requiring a specific
162 plasmid construct. Oligonucleotide primers used in these constructions are listed in Table 1 and
163 were obtained from Integrated DNA Technologies (Heverlee, Belgium). Constructs were
164 routinely transferred to *E. coli* DH5α by electroporation and verified by sequencing before further
165 use. The first plasmid construct was used to generate a *pyrE* deletion (locus tag SR42_16845) in
166 strain NTCT 11219. To this end, flanking loci of *pyrE* were cloned in pMTL84151 (31) to yield
167 pMTL84151_*pyr*5'*pyr*3'. More specifically, the 5' fragment (1086 bp) was amplified from
168 genomic DNA (gDNA) of NCTC 11219 with primers pyrE_5'F/pyrE_5'R and the 3' fragment
169 (1164 bp) with primers pyrE_3'F/pyrE_3'R. Amplicons were restricted with respectively
170 KpnI/BamHI, and BamHI/XhoI, ligated end-to-end in pMTL84151 opened with KpnI and XhoI,
171 and electroporated to *E. coli* DH5α. Secondly, the plasmid pMTL84151_Δ*bont*, used to replace
172 *bont/E* for *ermB* was constructed as illustrated in Fig. 1. Flanking loci (5' locus: 1080 bp; 3'
173 locus: 1261 bp) of *bont/E* were first cloned in pMTL84151, using primers bontE_5'F/bontE_5'R

and bontE_3'F/bontE_3'R for amplification. These loci include the first two codons of *bont*/E in the 5' fragment and the last sixteen codons in the 3' fragment. After restriction of the PCR products with respectively KpnI/BamHI and BamHI/XhoI, the 5' and 3' fragments were ligated end-to-end in pMTL84151 opened with KpnI and XhoI. Subsequently, *ermB* (amplified from pMTL82254 with pMTL82254ermB_F/pMTL82254ermB_R and restricted at both ends with BamHI) was inserted in the BamHI site of this plasmid, resulting in pMTL84151_*5'bont_ermB_3'bont*. In addition, wild type *pyrE* of NCTC 11219 (675 bp) was amplified from start to stop codon with primers pyrE11219_F/pyrE11219_R, restricted with NdeI/SacI and placed after promotor p_{fdx} in pMTL83353 opened with the same enzymes. p_{fdx} was used to drive *pyrE* expression because *pyrE* is located at the end of an operon in NCTC 11219 and thus could not be simply amplified with its native promotor. Hereafter, the fragment containing p_{fdx} and *pyrE* was amplified with pMTL83353_F/pyrE11219_R, digested with SbfI and SacI and cloned in pMTL84151_*5'bont_erm_3'bont*, restricted with the same enzymes. This resulted in the plasmid pMTL84151_Δ*bont*. A third plasmid was designated pMTL84151_WT*pyr*, used for restoring the *pyrE* deletion back to wild type. This was created by cloning a *pyrE* amplicon with its 5' and 3' flanking regions (2956 bp) generated with primers pyrE_5'F/pyrE_3'R and restricted with KpnI and XhoI, into pMTL84151, cleaved with the same enzymes. In this construct, a promotor is absent thus *pyrE* is not expressed on the plasmid.

Mating

Each of the four plasmid constructs described above was introduced in *E. coli* CA434 by electroporation and selecting Cm^R transformants. For plasmid transfer to *C. botulinum* NCTC 11219, one mL of an overnight LB culture of *E. coli* donor containing the appropriate plasmid was centrifuged, the pellet was washed with PBS (phosphate buffered saline, 8 g/l NaCl, 0.2 g/l

KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH adjusted with HCl to 7.4) and taken in the anaerobic workstation. Next, the pellet was resuspended in 200 µL of NCTC 11219 recipient grown for 24h in TPGY broth whereafter the mixture was spread on a cellulose acetate filter (poresize 0.45µm) on non-selective TYG agar. After 24h of incubation at 30 °C, the cells were washed off the filter with 1 mL PBS, and 200 µL was plated on selective RCM plates (Tm for resistance encoded on the plasmid, Cy against *E. coli* donor). Colonies appearing within 3 days were restreaked to purity on the same selective medium.

205 **Isolation and verification of *bont/E* ClosTron insertion mutant**

206 To confirm transfer of the ClosTron plasmid pMTL007C-E2:Cbo:*bontE*-211a to NCTC 11219,
207 Tm^R colonies were purified after mating and analysed by PCR using primers RAM_F/RAM_R.
208 Subsequently, transconjugants were streaked on RCM supplemented with Em, and well-
209 developed colonies appearing within 3 days were restreaked on the same medium for
210 purification. PCR and sequence analysis with primers bontE_F/bontE_R were performed to
211 verify successful intron insertion in *bont/E*. Individual colonies from a verified clone were then
212 screened for Tm sensitivity, indicating loss of the ClosTron plasmid, which was hereafter
213 confirmed by PCR using primers pCD6_F/pCD6_R.

214 To investigate whether only one insertion event took place, the Y-linker PCR described by Kwon
215 & Ricke (32) was used. In this method, gDNA of the ClosTron mutant is restricted to completion
216 with NlaIII and ligated to a Y-shaped linker, generated by mixing two oligonucleotides that are
217 complementary only at one end. The complementary parts of these oligonucleotides form the
218 stalk of the linker, which has a 3' CATG overhang that stocks to the NlaIII chromosomal DNA
219 ends. The non-complementary parts of the oligonucleotides form the Y-linker arms. PCR is then
220 conducted on the ligation mixture with a ClosTron specific primer (ClosTron_R) and a Y-linker
221 primer with a sequence identical, not complementary, to one of the arms. Only when the
222 ClosTron_R primer extends DNA synthesis during the first PCR cycle into the Y-linker arm, the
223 Y-linker primer can anneal and a PCR product will be formed. Consequently, the two primers
224 selectively amplify all fragments in the genome containing the ClosTron insert.

225

Isolation and verification of *bont/E* deletion mutant

After conjugation of pMTL84151-*pyr*5'*pyr*3' to NCTC 11219, transconjugants were streaked on RCM supplemented with 5-FOA to select for PyrE deficient mutants generated by double homologous recombination between the plasmid-based *pyrE*-flanking fragments and the corresponding chromosomal sequences. Deletion was confirmed by PCR and sequence analysis with primers pyrE_5'Fb/pyrE_3'Rb, which anneal outside the homologous fragments involved in recombination. Further, a clone from which the plasmid was cured, was isolated by screening for Tm sensitivity. Next, NCTC 11219 Δ*pyrE* was mated with *E. coli* CA434 containing pMTL84151_Δ*bont*. Transconjugants were streaked on RCM supplemented with Em and 5-FOA, to select for clones in which double homologous recombination as well as loss of the plasmid had occurred. Plasmid loss was confirmed by the Tm sensitivity of the clones. PCR and sequence analysis with primers upbontE_F/downbontE_R, annealing outside the homologous fragments, were performed to confirm that *bont/E* was deleted and replaced by *ermB*. Finally, the *pyrE* gene was restored to wild type by first conjugating pMTL84151-WT*pyr* to NCTC 11219 Δ*pyrE* Δ*bontE*::*ermB* and subsequently propagating one transconjugant in liquid TPGY medium for about 60 generations and subsequently plating on uracil-deficient medium. Only clones in which the chromosomal *pyrE* gene is restored by allelic exchange with the plasmid-based promotorless *pyrE* grow well on these plates. One such clone, confirmed by PCR and sequence analysis to be identical to the wild type in the *pyrE* locus and from which the plasmid was cured as explained before, was isolated and designated NCTC 11219 Δ*bontE*::*ermB*.

Mouse bioassays

Mouse bioassays were performed for detection of active botulinum toxin in clostridial cultures. The assays were performed with *bont/E* knockout strains NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*pyrE* Δ*bontE*::*ermB*, and with the corresponding positive control strains NCTC 11219 wild type and NCTC 11219 Δ*pyrE*, respectively. Supernatants from 5-day cultures of the strains in trypticase glucose yeast broth supplemented with trypsin at 30 °C were used for toxin testing. A seroneutralization test using anti-E antitoxin which specifically neutralizes the toxic effects of BoNT/E was included. Neutralized (i.e. mixed with antitoxin) and untreated filtered supernatants were injected intraperitoneally in mice for evaluating the toxicity. Two animals were used per sample. Mice were observed at regular intervals for a period of 4 days for signs of botulism (ruffling of the fur, hypotonic abdomen, wasp-waist, difficulty in breathing, weakness of the limbs, and total paralysis) or death. Typical paralysis and/or death of the mice with prevention of these effects by the administration of antitoxin establishes a positive test for BoNT/E.

Phenotypical analysis of wild type NCTC 11219 and NCTC 11219 *bont/E* mutants

Unstressed growth. Wild type *C. botulinum* NCTC 11219 and mutants *bontE*211a::CT and Δ*bontE*::*ermB* were inoculated from single colonies in 1 ml TPGY broth in triplicate and incubated for 24h at 30 °C. The stationary cultures were diluted $5x10^3$-fold in 50 mL TPGY ($=t_0$), and incubated at 30 °C. Cell numbers were determined by plate counting on TPGY agar every 2h during 16h and again 8h later.

Stressed growth. Overnight cultures of wild type and mutants were streaked in sixfold on RCM plates with different pH (5.7, 5.5, 5.2, 4.9; adjusted with HCl and measured before and after

autoclaving) or NaCl content (1.9%, 2.1%, 2.3%, 2.5%; taking into account that 0.5% NaCl is already present in RCM). The plates were incubated at 30 °C in an anaerobic workstation and colony formation was observed macroscopically for several days. Growth at lower temperatures was analysed in sixfold by streaking single colonies that were pregrown at 12 °C on RCM plates and incubating in AnaeroGen bags (Oxoid) at 8 °C and 12 °C. Colony formation was observed daily.

Heat resistance of spores. Spore crops were made in triplicate from different colonies per strain and used within two weeks for this experiment. The initial spore count was determined by plate counting on TPGY after a heat treatment to inactivate remaining vegetative cells (65 °C, 10 min), but non-preheated samples were used for the heat inactivation experiment. Heat treatments were conducted in a heating block at 70 °C, 73 °C, 90 °C and 93 °C. At different time points, samples were taken and cooled immediately to stop inactivation, diluted in 0.85% NaCl, plated on TPGY and incubated at 30 °C for colony counting. For the treatments at 90 °C and 93 °C, lysozyme (10 µg/ml; Carl Roth GmbH) was added to the plating medium.

Statistical analysis

To statistically assess the equivalence of unstressed growth, spore yield and spore heat resistance between the wild type and the two *bont/E* mutants, the two-tailed unpaired Student's t test was used with a significance level of 0.05. Since growth at low temperature was analysed in a ‘time to colony formation’ experiment, in this case a logistic regression model was fitted using the day, temperature and strain as predictor variables. The proposed model was highly significant (P-value whole model likelihood ratio test < 0.001; generalized $R^2 = 0.9063$) and showed no lack of fit (P-

290 value lack of fit likelihood ratio test ~ 1), indicating it was suitable for testing the individual
291 parameters.

RESULTS AND DISCUSSION

C. botulinum NCTC 11219 is a group II type E strain isolated in 1979 from salmon after an outbreak of human botulism (33), of which we previously reported the genome sequence in four contigs (accession number JXMR01000001-JXMR01000004, 34). Here, we used this strain for the construction of atoxigenic mutants. The ClosTron system was used for insertional inactivation of the *bont/E* gene, whereas a new approach based on double homologous recombination using the selection markers *pyrE* and *ermB* was designed to create a *bont/E* deletion.

Construction of insertion mutant *C. botulinum* NCTC 11219 *bontE*211a::CT

Plasmid pMTL007C-E2:Cbo:*bontE*-211a, containing an intron retargeted to insert in *bont/E,* was transferred by conjugation to NCTC 11219. Since the intron contains a retrotransposition-activated marker (RAM) based on the *ermB* gene, successful insertion of the intron in the genome is selectable by the expression of Em resistance. Em^R clones were picked up and analysed by PCR and sequence analysis. The intron was correctly located in one clone at position 211 in the *bont/E* open reading frame with an antisense orientation of *ermB* relative to *bont/E* (Fig. 2, Fig. 3 lane 2-3). Further analysing this clone by Y-linker PCR and sequencing of the PCR product, confirmed that only a single intron was present in the genome. The efficiency of the different steps in the ClosTron mutagenesis procedure was rather low. For conjugation of the ClosTron plasmid to NCTC 11219, several attempts were needed to obtain only two transconjugants. Similarly, several clones of transconjugants had to be restreaked to obtain 15 Em^R colonies, and only one of these had the correct intron insertion. This confirms earlier statements in the literature that *C. botulinum* is less genetically tractable than most other clostridia (21, 35).

Construction of deletion mutant *C. botulinum* NCTC 11219 Δ*bontE*::*ermB*

A deletion strategy to knockout *bont/E* was developed by combining different genetic tools that have not yet been used in gIICb, despite their success in other clostridia. Since effective suicide plasmids have not yet been described for *C. botulinum,* and given the reported low frequency of DNA transfer into gIICb, we chose to make use of pseudo-suicide plasmids for conjugation (30, 36). These plasmids autonomously replicate but are segregationally unstable, providing the advantage to be lost more rapidly in the absence of selection after successful conjugation. A set of modular shuttle vectors, the pMTL80000 series, carrying four different gram-positive replicons was constructed by Heap *et al.* (2009) for use in the clostridia. We selected pMTL84151 with the pCD6 replicon from this set since this plasmid has the lowest stability in groups I and II *C. botulinum* (31, unpublished results). Another key element in the knockout strategy was the use of the *pyrE* gene as a bidirectional selection marker. First, *pyrE* was deleted from NCTC 11219 resulting in a 5-FOA resistant and uracil auxotroph strain (Fig. 3. Lane 4-5). Then the obtained Δ*pyrE* mutant was used as background to delete *bont/E* and replace it with an *ermB* marker. This was achieved by first conjugating pMTL84151_Δ*bont* (Fig. 1) to the Δ*pyrE* mutant and selecting for Tm resistance, then streaking a transconjugant on medium containing both Em and 5-FOA. Since the plasmid expresses *pyrE in trans*, 5-FOA selects for its loss. Further, clones that lost the plasmid can only remain resistant to Em when the *ermB* gene was exchanged with the *bont/E* gene by double homologous recombination. Although this procedure selects for two events simultaneously, the frequency was sufficient to allow isolation of several colonies from a single streak. Obviously, the efficiency benefits from the low segregational stability of the plasmid. Loss of the plasmid was confirmed by loss of Tm resistance, and PCR and sequence analysis confirmed the replacement of *bont/E* by *ermB* (Fig. 2, Fig. 3 lane 6-7).

Finally, the *pyrE* deletion was restored to wild type by swapping *pyrE* and its flanking regions back to the chromosome by double homologous recombination with pMTL84151_WT*pyr*. To this end, the plasmid was first conjugated to NCTC 11219 Δ*pyrE* Δ*bontE*::*ermB*. The Tm^R transconjugants remained resistant to 5-FOA, which confirmed that *pyrE* was not expressed on pMTL84151_WT*pyr*. Since *pyrE* was cloned without its promotor (which is further upstream the *pyr* operon) on this plasmid, this result indicates that there is no leaky expression from plasmid promoters. In line with this observation, the transconjugants did not grow when they were plated on uracil-deficient medium, except for some rare colonies which showed normal growth when restreaked on the same medium. From these, subclones having lost the plasmid with a Tm^S phenotype were isolated. These were confirmed to have a wild type chromosomal *pyrE* allele, and were designated as NCTC 11219 Δ*bontE*::*ermB*.

Mouse bioassays

The presence of BoNT/E in culture supernatants of the NCTC 11219 wild type and the Δ*pyrE* strain was confirmed whereas no active toxin was detected for the *bont/E* insertion and deletion mutants. All mice injected with supernatant of wild type and Δ*pyrE* died within a day, while mice injected with supernatants of the mutants or supernatants from the *bont/E* wild type strains mixed with antitoxin E, survived till the end of observation at 4 days. Thus, loss of toxicity in both mutants was confirmed. In addition, the seroneutralization test using anti-E antitoxin confirmed that only one neurotoxin is produced by NCTC 11219, as was already predicted from the genome sequence (34).

Phenotypical analysis of wild type NCTC 11219 and NCTC 11219 *bont/E* mutants

Nontoxic mutants of gIICb have the potential to greatly facilitate basic research and food challenge studies with this organism, provided that their properties related to growth, sporulation, survival and inactivation are not modified due to the knockout of the *bont/E* gene. Here, we compared the growth, sporulation and spore heat resistance of both constructed *bont/E* mutants and the wild type NCTC 11219 strain.

Unstressed growth. The growth curves of the mutants and the wild type at 30 °C were very similar (Fig. 4). The DMFit software was used to determine the maximum growth rate μ_{max} and the upper asymptote y_{end}. The lag phase could not be evaluated because there were not enough points available to measure this accurately. No significant differences existed between μ_{max} and y_{end} of the wild type, ClosTron and Δ*bontE* mutant. The values were 0.7 +/- 0.2, 0.5 +/- 0.1, 0.4 +/- 0.0 (log CFU/ml)/h for μ_{max}, and 7.8 +/- 0.3, 8.0 +/- 0.0; 8.0 +/- 0.1 log CFU/ml for y_{end}, respectively.

Stressed growth. A quantitative comparison of growth under acidic, NaCl and low temperature stress was performed by assessing the time to colony formation on RCM plates. On acidified RCM, results of both mutants and wild type were identical in sixfold. Single colonies of 1 mm were visible after 24 h at pH 5.7 and after 30 h at pH 5.5, while pinpoint colonies appeared after 72 h at pH 5.2. No colonies were formed at pH 4.9 up to 5 days. Under NaCl stress, all three strains showed clear single colonies in sixfold after 24 h at 1.9% and 2.1% NaCl. Unexpectedly, a small difference was observed at 2.3% NaCl between the Δ*bontE*::*ermB* strain compared to the wild type and ClosTron mutant. Strain Δ*bontE*::*ermB* formed clear 1 mm colonies after 24 h (sixfold) while those of the wild type and ClosTron mutant were pinpoint sized, and reached 1

mm only after 48 h (sixfold). No colonies appeared on 2.5% NaCl for any strain. The third stress condition tested, was growth at low temperature. Single colonies grown at 12 °C on RCM were restreaked in sixfold on RCM, then incubated at 12 °C and 8 °C, and the time to formation of colonies (1 mm) was registered. As opposed to the data for acid and NaCl stress, the time to colony formation at low temperature showed variability among the six replicates, as indicated in Table 2. A logistic regression model was fit on this data and showed that there was no significant effect of the strain (P-value effect likelihood ratio test 0.2388). Therefore, it can be concluded that there is no significant difference in growth at low temperature between the three strains.**Spore production.** Sporulation was performed in two-phase sporulation medium and observed regularly with phase contrast microscopy. Spore formation started within 72h for all strains. Spore counts were determined after 6 days by plate counting after inactivation of the vegetative cells at 65 °C for 10 min. No differences were observed in spore yield between wild type, *bontE*211a::CT and Δ*bontE*::*ermB*. Spore crops used for treatments at 90 °C/93 °C had a yield of 7.8 log +/- 0.1 CFU/ml, 8.1 +/- 0.3 CFU/ml and 7.9 +/- 0.1 CFU/ml, respectively. The spore crops used for heat treatments at 70 °C/73 °C all had the same yield, 7.6 log +/- 0.1 CFU/ml.

Spore heat resistance. In the analysis of spore heat resistance, we incorporated lysozyme in the recovery medium because this is known to increase the number of survivors of gIICb spores (37, 38). This is because the cortex hydrolases which are required for spore germination are very heat sensitive, but can be substituted by exogenous lysozyme. The inactivation curves at 90 °C and 93 °C showed a biphasic trend, with an initial rapid decrease of about 2 – 2.5 log, followed by a slower log-linear decrease (Fig. 5). This is in line with previous reports, and has been attributed to the existence of a lysozyme-impermeable and -permeable fraction in the spore population (38,

403 39). The decimal reduction times (D-values) were calculated only from the log-linear part,
404 corresponding to the presumed lysozyme-permeable fraction. No differences were observed in
405 the $D_{90°C}$ and $D_{93°C}$ between the three strains, suggesting that spore heat resistance is not affected
406 by inactivation of *bontE*. In addition, we chose to report heat resistance without lysozyme in the
407 recovery medium. This reduces the apparent heat resistance of the spores, and treatments were
408 therefore performed at 70 °C and 73 °C. Inactivation was log-linear in this case (Fig. 6), and
409 presumably correlates with the inactivation of the spore cortex hydrolases. The $D_{70°C}$ of the two
410 mutants was slightly but significantly lower than that of wild type, but no difference was found
411 between the $D_{70°C}$ of both mutants mutually. In contrast, the $D_{73°C}$ was not different between the
412 three strains. Together, these results indicate that spore heat resistance is unaffected in the
413 mutants, except for a small difference at 70 °C. Since this difference was observed both in the
414 insertion and deletion mutant, it is unlikely to result from an accidental mutation unrelated to the
415 knockout of the *bont/E* gene. One possible explanation is that the effect is due to a polar effect on
416 one or more genes upstream or downstream of *bont/E*. However, given the gene context as shown
417 in Fig. 2, it is difficult to imagine how the same polar effect could exist in both mutants. Finally,
418 it cannot be excluded that the difference is a direct consequence of the abolition of toxin
419 production. For example, it has been recently demonstrated that there is a regulatory link between
420 sporulation and toxin production in gIICb strain Beluga via the Spo0A gene product (40).

421 In conclusion, two strategies were employed in this study for obtaining a gIICb surrogate in
422 which the *bont/E* gene is inactivated. Besides the well-known ClosTron system which generates
423 insertional knockouts, we additionally developed a new approach based on double homologous
424 recombination for the replacement of *bont/E* with an *ermB* cassette. Growth under unstressed and
425 stressed conditions, sporulation and spore heat resistance of both mutants were compared with

426 the wild type NCTC 11219 and were unaffected, except for two properties where small
427 differences were noted. Heat resistance of the spores at 70 °C was slightly lower for both
428 mutants. In addition, growth of the deletion mutant in 2.3% NaCl was faster than for the other
429 strains. The reason for the changes is currently unclear, and it could be of interest to investigate
430 whether they are related to the loss of toxin production or to random mutations that have occurred
431 during strain construction. However, since the deviations in the mutants are small compared to
432 the natural strain-to-strain variation within gIICb, the nontoxic strains will be useful to
433 investigate the safety of novel food processing and preservation techniques and for food
434 challenge studies. Furthermore, the method for gene deletion developed in this work is a novel
435 tool to construct nontoxic derivatives of type B and F gIICb strains as well as to inactivate any
436 other gene in gIICb and other clostridia.

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547 Presented at 52nd Interagency Botulism Research Coördinating Committee, Frederick, Maryland,
548 USA, 25 to 28 October 2015.
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FIGURES AND TABLES

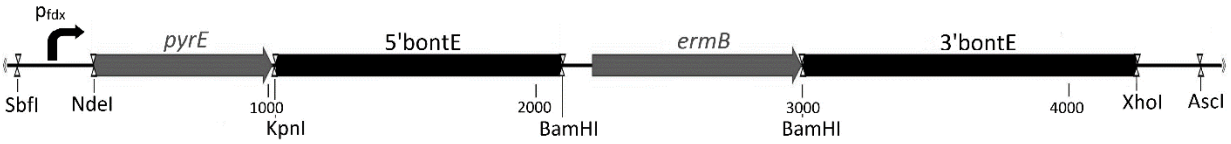
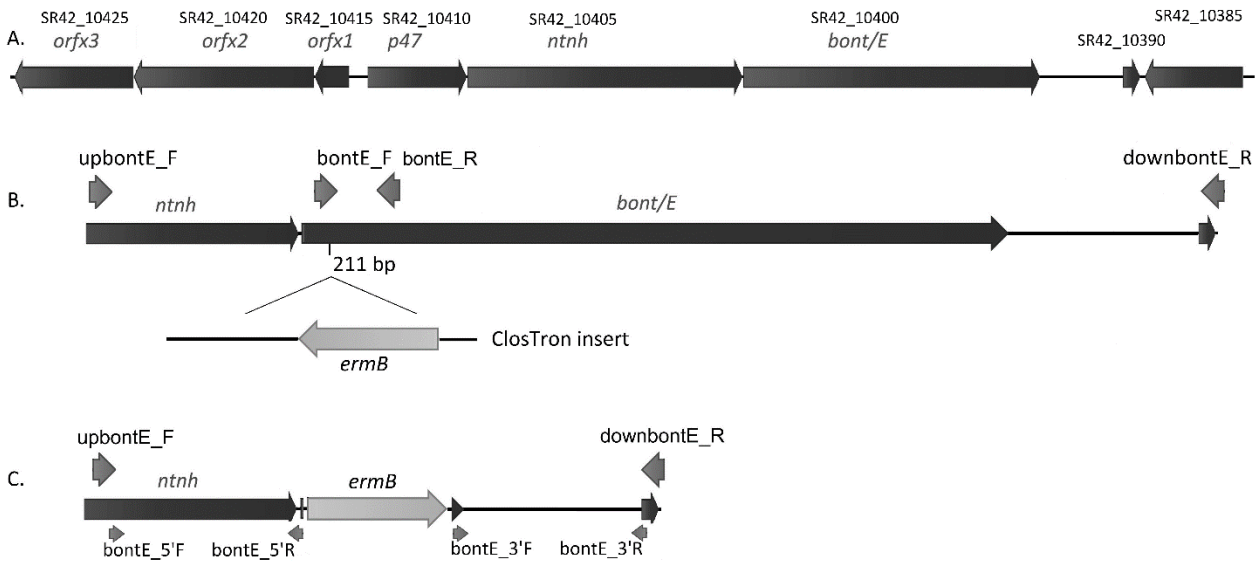


Fig. 1: Scheme showing the different fragments cloned between SbfI and AscI restriction sites of shuttle vector pMTL84151 (Tm^R) to create pMTL84151_Δ*bont* (Tm^R Em^R), that was used for replacing the *bont/E* gene for an *ermB* marker. The p_{fdx} promotor (bended arrow) was derived from pMTL83353 and drives expression of *pyrE*, which was amplified from gDNA of NCTC 11219. The 5' and 3' fragments of *bont/E* were also amplified from NCTC 11219 gDNA, and flank *ermB* derived from pMTL82254.



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565 Fig. 2: Schematic representation of A) *bont/E* with its upstream and downstream genes in *C.*
566 *botulinum* NCTC 11219. Locus tags for all genes are also shown. B) Position of the ClosTron
567 insert after 211th base of *bont/E* open reading frame in NCTC 11219 *bontE*211a::CT, with *ermB*
568 antisense relative to *bont/E*. Also shown are primers bontE_F/bontE_R, annealing up- and
569 downstream of the ClosTron target site, and primers upbontE_F/downbontE_R, annealing outside
570 the loci used in double homologous recombination to delete *bont/E*. C) NCTC 11219
571 Δ*bontE*::*ermB*, with *bont/E* deleted and replaced by *ermB*, confirmed by PCR using primers
572 upbontE_F/downbontE_R. The position of the cloning primers bontE_5'F/bontE_5'R and
573 bontE_3'F/bontE_3'R, used for construction of pMTL84151_Δ*bont*, is also indicated.

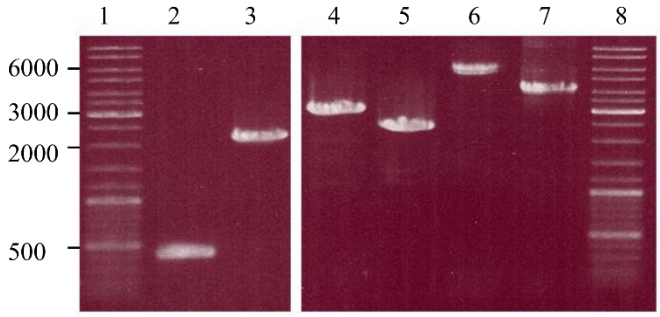


Fig. 3: PCR analysis of different strain constructs. Lane 1 and 8: Molecular weight marker GeneRuler from Life Technologies; lane 2: NCTC 11219, *bont/E* fragment amplified with primers bontE_F/bontE_R (expected size: 410 bp); lane 3: NCTC 11219 *bontE*211a::CT, *bont/E* fragment with ClosTron insertion amplified with primers bontE_F/bontE_R (expected size: 2008 bp); lane 4: NCTC 11219, *pyrE* region amplified with primers pyrE_5Fb/pyrE_3Rb (expected size: 3174 bp); lane 5: NCTC 11219 Δ*pyrE*, *pyrE* region amplified with primers pyrE_5Fb/pyrE_3Rb (expected size: 2474 bp); lane 6: NCTC 11219 Δ*pyrE*, *bont/E* region amplified with primers upbontE_F/downbontE_R (expected size: 6218 bp); lane 7: NCTC 11219 Δ*bontE*::*ermB*, *bont/E* region amplified with primers upbontE_F/downbontE_R (expected size: 3449 bp).

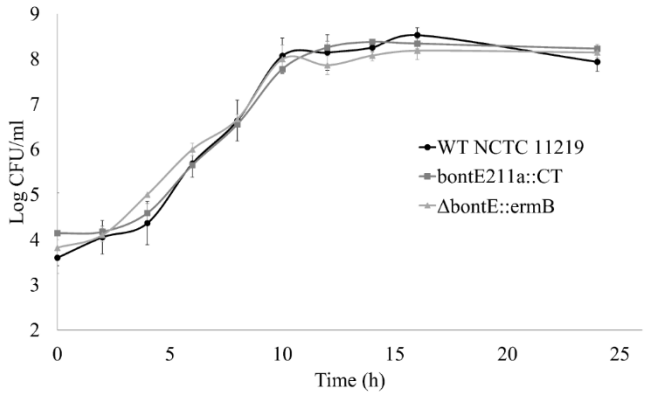
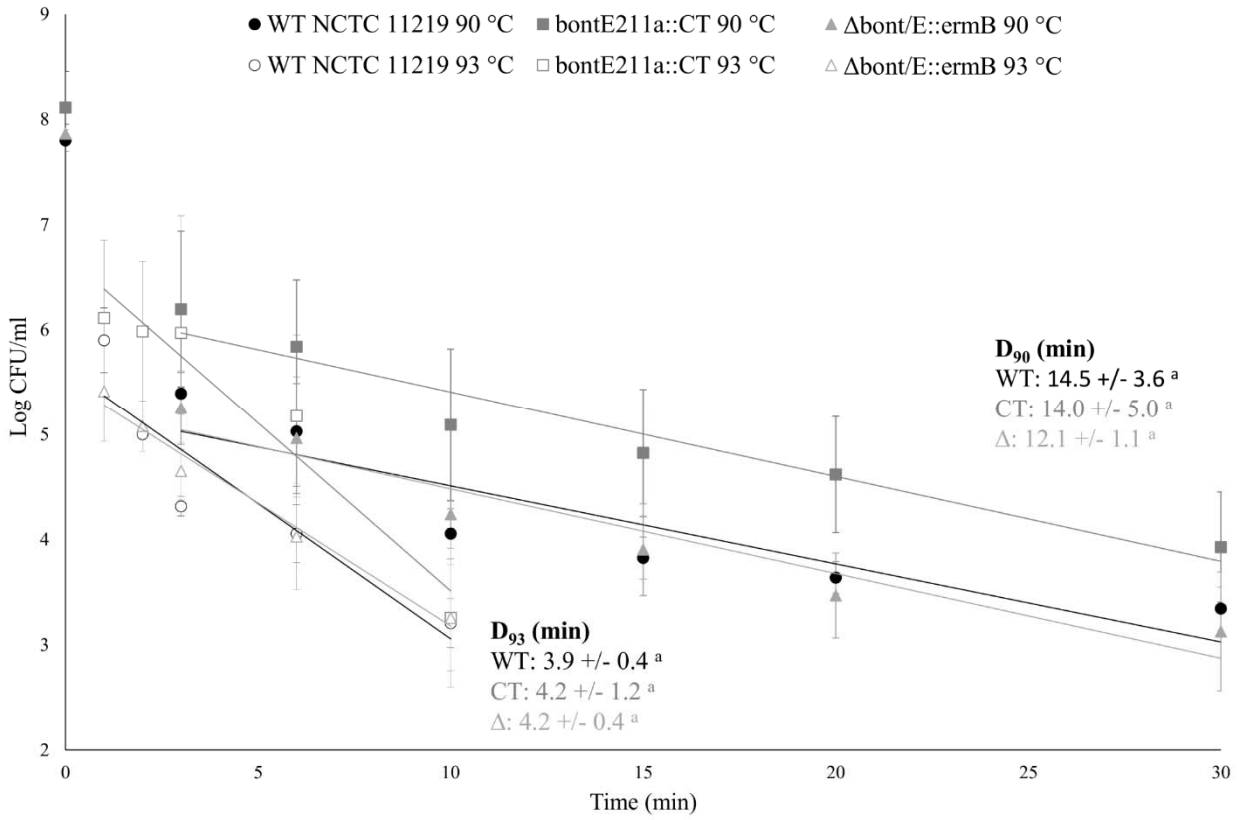


Fig. 4: Growth in TPGY broth at 30 °C of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* determined by plate counting on TPGY. Averages +/- standard deviations are shown from three independent experiments.



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593 Fig. 5: Heat inactivation of spores of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and
594 NCTC 11219 Δ*bontE*::*ermB* at 90 °C and 93 °C. Averages +/- standard deviation of three
595 independent experiments are shown, as well as average D-values +/- standard deviation at 90 °C
596 and 93 °C derived from the linear part of the curve. A common letter superscript at the same
597 temperature indicates that there is no significant difference.

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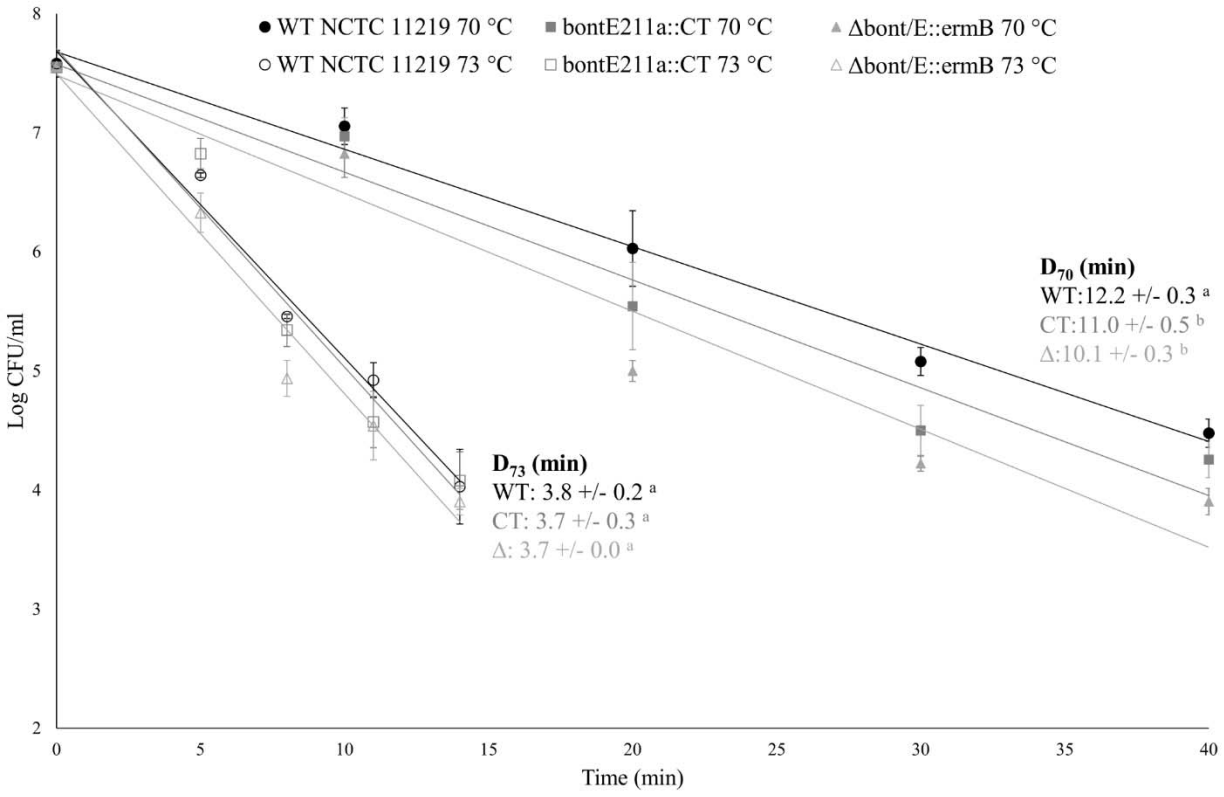


Fig. 6: Heat inactivation of spores of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* at 70 °C and 73 °C. Averages +/- standard deviation of three independent experiments are shown, as well as average D-values +/- standard deviation at 70 °C and 73 °C. A common letter superscript at the same temperature indicates that there is no significant difference. Since tailing occurred after 40 min at 70 °C and after 15 min at 73 °C, the corresponding data is not shown or used.

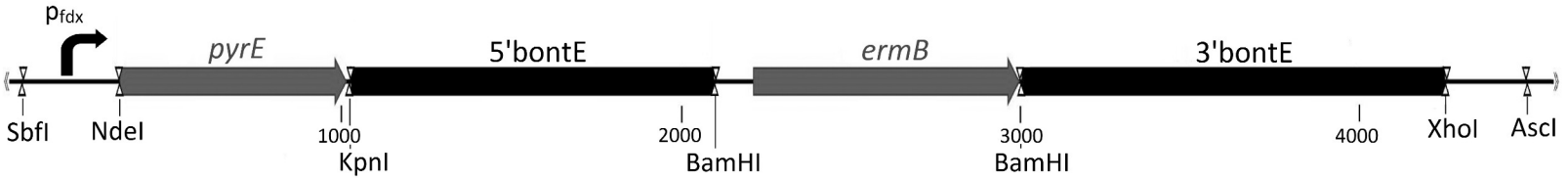
609 Table 1: PCR oligonucleotides used for cloning and construct verification. Restriction sites are
610 underlined: KpnI (GGTACC), BamHI (GGATCC), XhoI (CTCGAG), NdeI (CATATG), SacI
611 (GAGCTC) and SbfI (CCTGCAGG). The primers' coordinates of NCTC 11219 are indicated in
612 the third column, based on accession numbers JXMR01000001[a] or JXMR01000004[b].

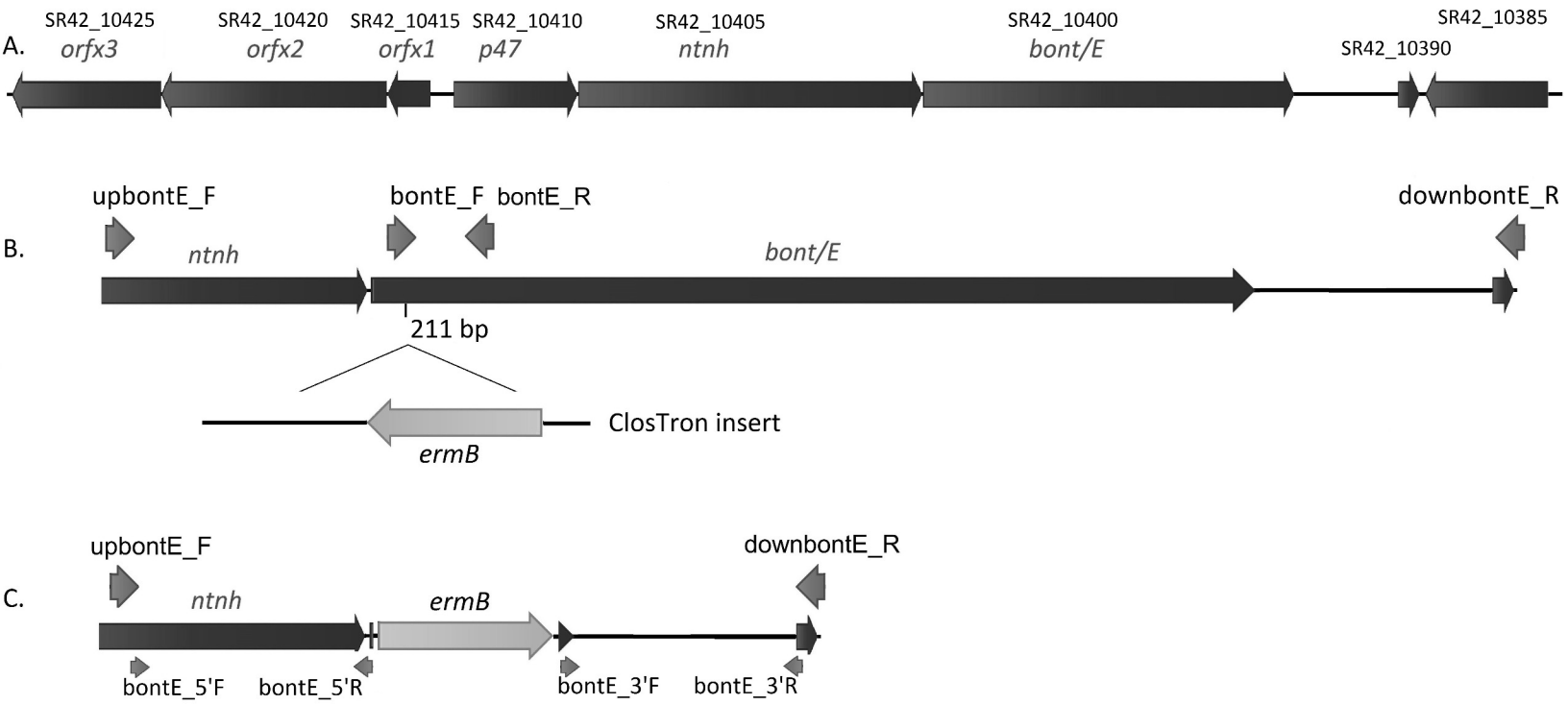
Name	**Sequence (5'-3')**	**Coordinate of 3' end**
ClosTron		
bontE_F	CCAGGCGGTTGTCAAGAATTTTAT	2298420[a]
bontE_R	TCAAATAAATCAGGCTCTGCTCCC	2298057[a]
RAM_F	ACGCGTTATATTGATAAAAATAATAATAGTGGG	
RAM_R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG	
pCD6_F	GTTGGGAGTAGTTGTGC	
pCD6_R	ATGGTATCTCATTATTGGC	
ClosTron_R	GTTTCAGACACTTTCCTCTATCGAG	
Y-linker primer	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG	
ΔpyrE		
pyrE_5'F	AACGGTACCCCACTACGTTCTCTCTTAGAGG	246521[b]
pyrE_5'R	AACGGATCCTGAGTTTAAGGTTTTTAGTTGG	247564[b]
pyrE_3'F	CATGGATCCTCCTCCCAAATTTCATAATGT	248312[b]
pyrE_3'R	CATCTCGAGTGTTTTATGCTGTGGTCCTG	249436[b]
pyrE_5'Fb	CCAACTTTATCAAAAGCTCAG	246369[b]
pyrE_3'Rb	CTAATACAGGAAAGCATGGC	249503[b]
pMTL84151_mcsF	AGGAAACAGCTATGACCG	
pMTL84151_mcsR	GACGTTGTAAAACGACGG	
ΔbontE		
pyrE11219_F	AGGCATATGGAAGCATATAAAAAAGAG	248264[b]
pyrE11219_R	CTTGAGCTCCTACTTAGCACCATATTC	247627[b]
bontE_5'F	TAAGGTACCGTTATTGGAGATACATCCGG	2299564[a]
bontE_5'R	TAAGGATCCTGGCATATACAGCATCTCC	2298522[a]
bontE_3'F	TAAGGATCCAGAGATCATACAAACAGCAATGG	2294800[a]
bontE_3'R	TAACTCGAGCTATTCGAGAGAGCATTGGTCG	2293583[a]
upbontE_F	GAACTACTTATAAAAGAACAAACCTCACC	2299627[a]
downbontE_R	GAATGGATATCTTAGGATAATCATTCCAC	2293466[a]
pMTL83353_F	GAGCCTGCAGGATAAAAAAATTGTAG	
pMTL82254ermB_F	GTTGGATCCGAAGCAAACTTAAGAGTGTG	

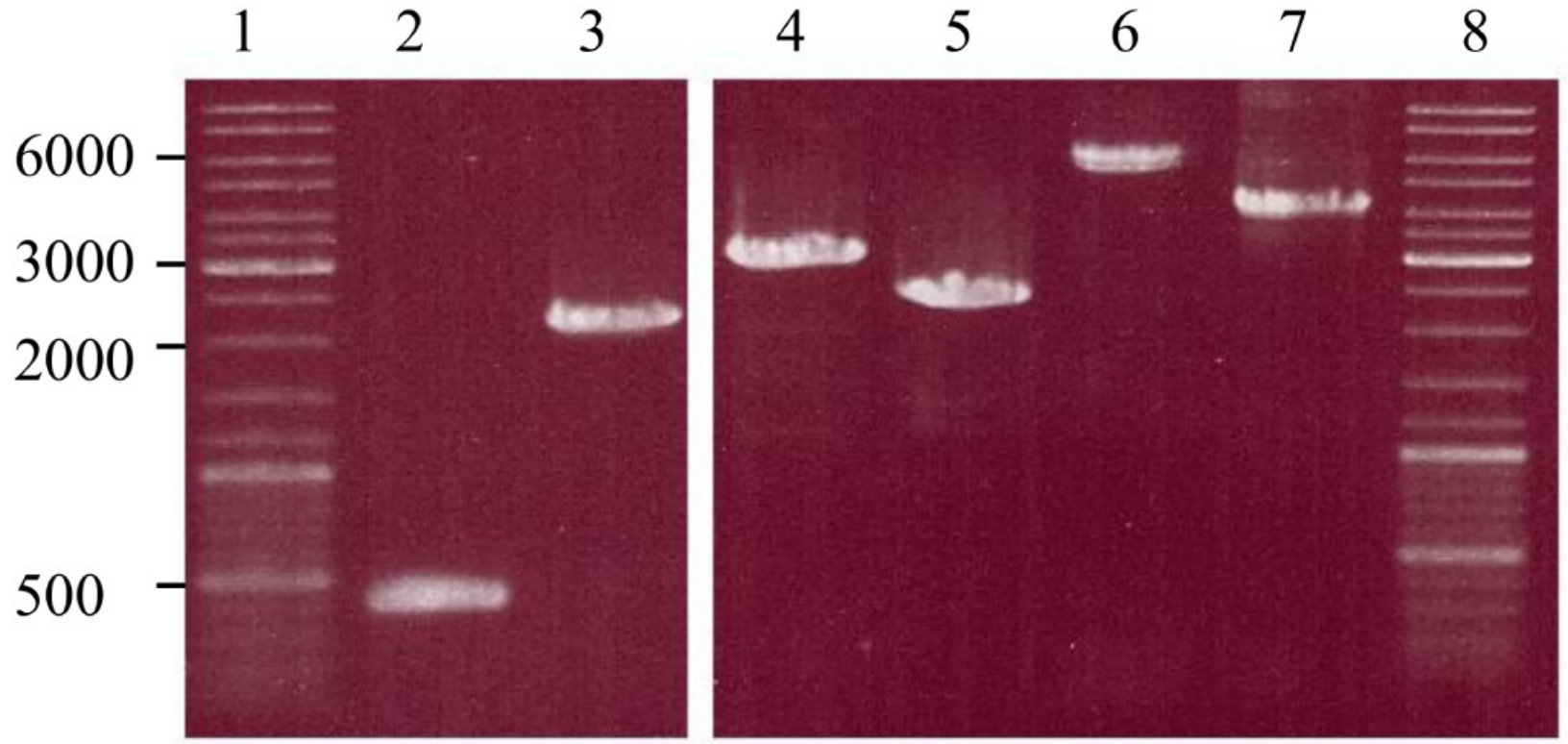
pMTL82254ermB_R TACGGATCCACATTCCCTTTAGTAACGTG

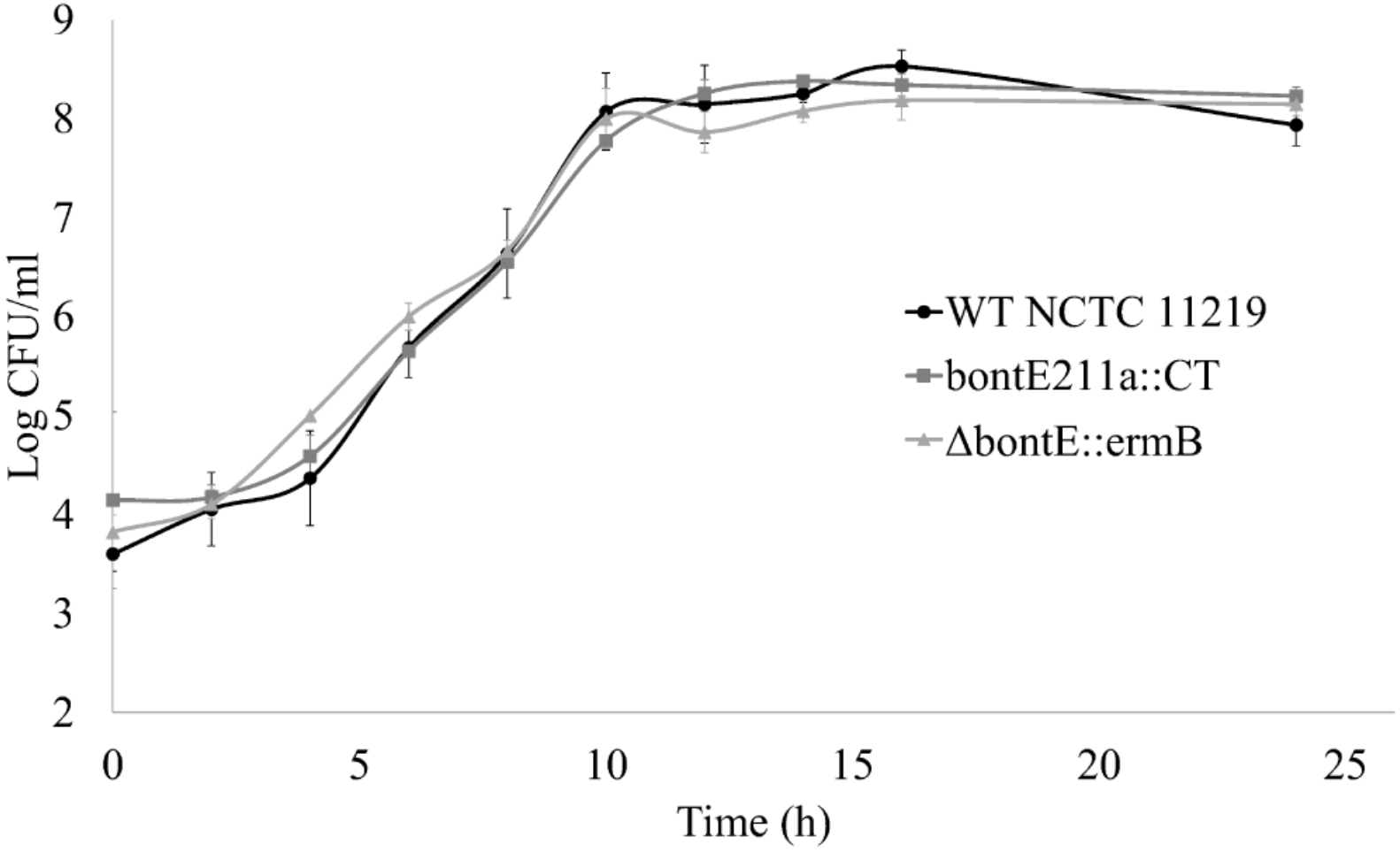
Table 2: Growth at 12 °C and 8 °C of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* on RCM plates. Single colonies were restreaked in sixfold and the time to formation of 1 mm colonies was noted.

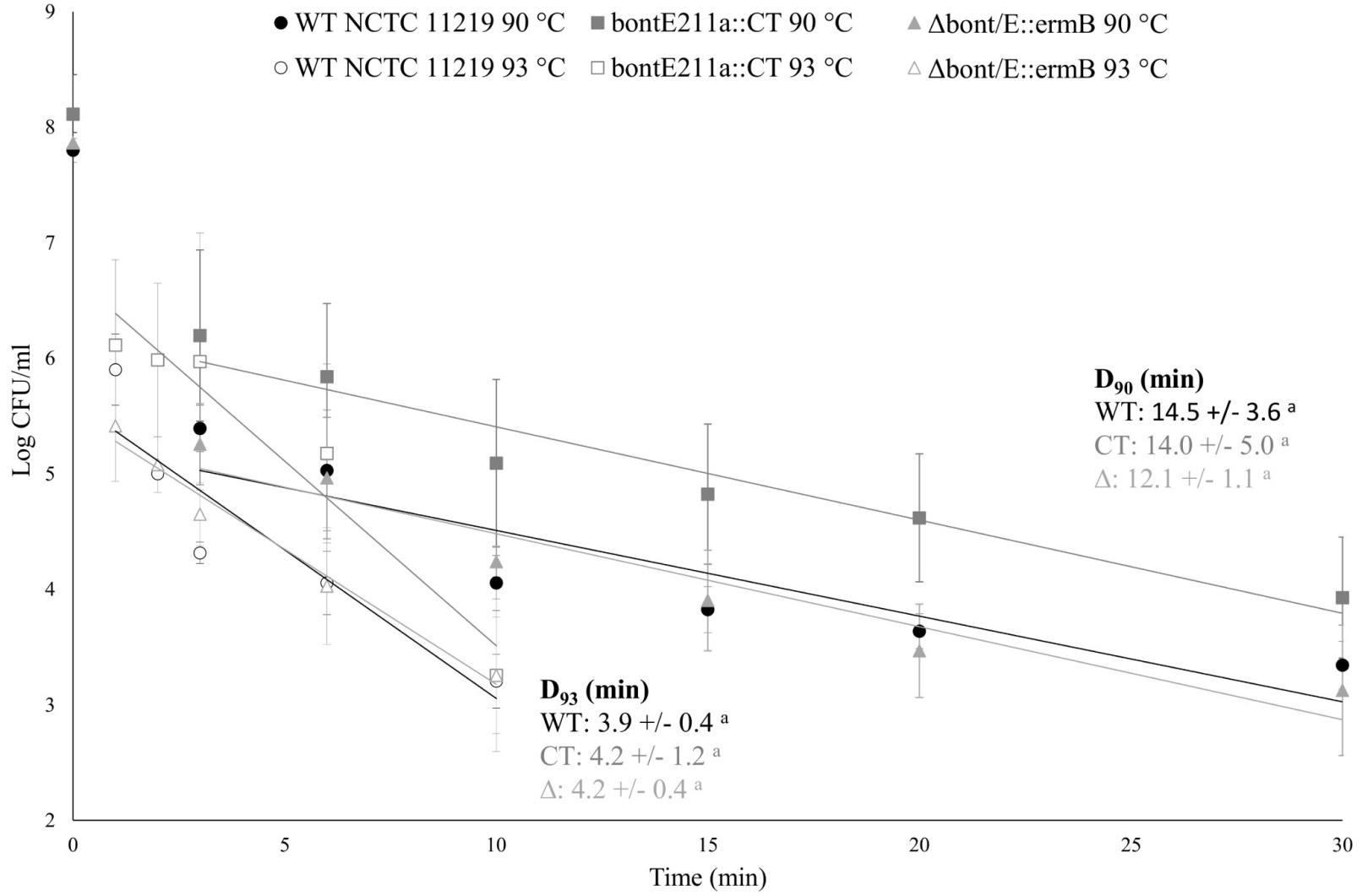
	Number of plates showing single colonies				
	at 12 °C		**at 8 °C**		
	3 days	**4 days**	**7 days**	**8 days**	**9 days**
NCTC 11219	5/6	6/6	4/6	5/6	6/6
*bontE*211a::CT	4/6	6/6	4/6	6/6	6/6
Δ*bontE*::*ermB*	3/6	6/6	3/6	4/6	6/6

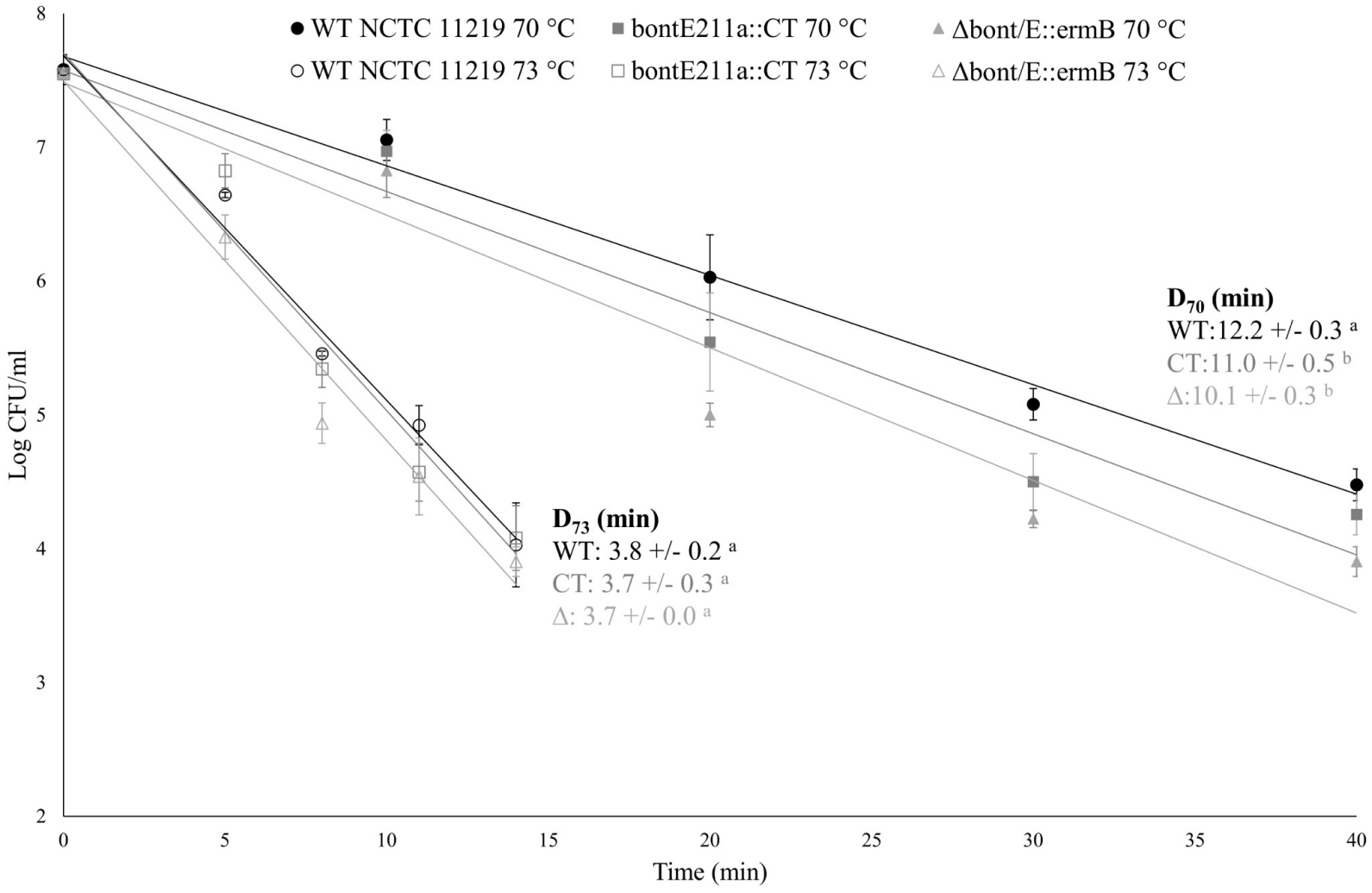

pfdx
pyrE
5'bontE
ermB
3'bontE
SbfI
NdeI
1000
KpnI
2000
BamHI
3000
BamHI
4000
XhoI
AscI


A.
SR42_10425
orfx3
SR42_10420
orfx2
SR42_10415
orfx1
SR42_10410
p47
SR42_10405
ntnh
SR42_10400
bont/E
SR42_10390
SR42_10385
B.
upbontE_F
bontE_F
bontE_R
downbontE_R
ntnh
bont/E
211 bp
ClosTron insert
ermB
C.
upbontE_F
downbontE_R
ntnh
ermB
bontE_5'F
bontE_5'R
bontE_3'F
bontE_3'R


1
2
3
4
5
6
7
8
6000
3000
2000
500


9
8
7
6
5
4
3
2
Log CFU/ml
0
5
10
15
20
25
Time (h)
WT NCTC 11219
bontE211a::CT
ΔbontE::ermB


● WT NCTC 11219 90 °C
■ bontE211a::CT 90 °C
▲ Δbont/E::ermB 90 °C
○ WT NCTC 11219 93 °C
□ bontE211a::CT 93 °C
△ Δbont/E::ermB 93 °C
Log CFU/ml
Time (min)
D90 (min)
WT: 14.5 +/- 3.6 a
CT: 14.0 +/- 5.0 a
Δ: 12.1 +/- 1.1 a
D93 (min)
WT: 3.9 +/- 0.4 a
CT: 4.2 +/- 1.2 a
Δ: 4.2 +/- 0.4 a


WT NCTC 11219 70 °C
bontE211a::CT 70 °C
Δbont/E::ermB 70 °C
WT NCTC 11219 73 °C
bontE211a::CT 73 °C
Δbont/E::ermB 73 °C
D70 (min)
WT:12.2 +/- 0.3 a
CT:11.0 +/- 0.5 b
Δ:10.1 +/- 0.3 b
D73 (min)
WT: 3.8 +/- 0.2 a
CT: 3.7 +/- 0.3 a
Δ: 3.7 +/- 0.0 a
Log CFU/ml
Time (min)
8
7
6
5
4
3
2
0
5
10
15
20
25
30
35
40

Name	Sequence (5'-3')	Coordinate of 3' end
ClosTron		
bontE_F	CCAGGCGGTTGTCAAGAATTTTAT	2298420[a]
bontE_R	TCAAATAAATCAGGCTCTGCTCCC	2298057[a]
RAM_F	ACGCGTTATATTGATAAAAATAATAATAGTGGG	
RAM_R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG	
pCD6_F	GTTGGGAGTAGTTGTGC	
pCD6_R	ATGGTATCTCATTATTGGC	
ClosTron_R	GTTTCAGACACTTTCCTCTATCGAG	
Y-linker primer	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG	
Δ*pyrE*		
pyrE_5'F	AACGGTACCCCACTACGTTCTCTCTTAGAGG	246521[b]
pyrE_5'R	AACGGATCCTGAGTTTAAGGTTTTTAGTTGG	247564[b]
pyrE_3'F	CATGGATCCTCCTCCCAAATTTCATAATGT	248312[b]
pyrE_3'R	CATCTCGAGTGTTTTATGCTGTGGTCCTG	249436[b]
pyrE_5'Fb	CCAACTTTATCAAAAGCTCAG	246369[b]
pyrE_3'Rb	CTAATACAGGAAAGCATGGC	249503[b]
pMTL84151_mcsF	AGGAAACAGCTATGACCG	
pMTL84151_mcsR	GACGTTGTAAAACGACGG	
Δ*bontE*		
pyrE11219_F	AGGCATATGGAAGCATATAAAAAAGAG	248264[b]
pyrE11219_R	CTTGAGCTCCTACTTAGCACCATATTC	247627[b]
bontE_5'F	TAAGGTACCGTTATTGGAGATACATCCGG	2299564[a]
bontE_5'R	TAAGGATCCTGGCATATACAGCATCTCC	2298522[a]
bontE_3'F	TAAGGATCCAGAGATCATACAAACAGCAATGG	2294800[a]
bontE_3'R	TAACTCGAGCTATTCGAGAGAGCATTGGTCG	2293583[a]
upbontE_F	GAACTACTTATAAAAGAACAAACCTCACC	2299627[a]
downbontE_R	GAATGGATATCTTAGGATAATCATTCCAC	2293466[a]
pMTL83353_F	GAGCCTGCAGGATAAAAAAATTGTAG	
pMTL82254ermB_F	GTTGGATCCGAAGCAAACTTAAGAGTGTG	
pMTL82254ermB_R	TACGGATCCACATTCCCTTTAGTAACGTG	

Number of plates showing single colonies

	at 12 °C		at 8°C		
	3 d	**4 d**	**7 d**	**8 d**	**9 d**
NCTC 11219	5/6	6/6	4/6	5/6	6/6
bontE211a::CT	4/6	6/6	4/6	6/6	6/6
ΔbontE::ermB	3/6	6/6	3/6	4/6	6/6